Mammoth Project Report

# Summary

This report summarizes a series of studies focused on the formulation, characterization, and analysis of therapeutic proteins, primarily immunoglobulin G (IgG). The work encompassed the development and validation of analytical methods, screening of excipients to improve formulation characteristics, and fabrication and testing of microsphere-based delivery systems.

Key analytical methods, including BCA, ELISA, and dot blot assays, were established to quantify protein concentration and activity. The BCA assay was validated for use with PLGA microspheres, demonstrating minimal interference from the polymer matrix. Dot blot assays confirmed that the encapsulation process had a minimal negative impact on antibody activity, although challenges with non-specific binding were identified, indicating a need for further optimization.

Extensive screening of numerous excipients was conducted to enhance protein solubility, stability, and loading efficiency. Trehalose was identified as a superior excipient to lactose for a mammoth protein formulation, significantly increasing solubility by 20% and reducing the degradation rate. In studies evaluating IgG loading into blank microspheres, several excipients, including chitosan, lecithin, and polyallylamine, substantially improved loading percentages. A broad solubility screen of potential additives in various solvents highlighted significant variability, with some compounds failing to dissolve under the tested conditions.

The fabrication of microspheres was investigated by varying polymer concentrations, which directly influenced particle size, uniformity, porosity, and yield. While microsphere properties could be effectively tuned, attempts to passively load hIgG into pre-formed microspheres via incubation were unsuccessful, resulting in negligible uptake. The findings provide a foundational understanding of key parameters influencing biologic formulations, identify promising excipients, and validate analytical techniques for future development.

# Introduction

The development of long-acting formulations for therapeutic antibodies, such as Immunoglobulin G (IgG), is critical for improving treatment regimens and patient compliance. This work focuses on creating a formulation capable of releasing 500 mg of IgG over a three-month period. Significant technical challenges include the limited aqueous solubility of IgG at high concentrations and the difficulty of achieving efficient loading into polymer-based delivery systems. The development process requires robust and validated analytical methods to accurately quantify protein concentration, encapsulation efficiency, and retained biological activity.

A series of experiments were conducted to address these challenges. The work involved a systematic evaluation of various excipients and additives to enhance both the solubility of IgG and its loading capacity into pre-formed polymer microspheres. Different microsphere formulations were constructed using polymers such as PLGA and Innocore, with adjustments to process parameters to manipulate physical characteristics like porosity and density. A post-loading methodology was investigated as the primary means of encapsulation. Concurrently, significant effort was dedicated to the development, optimization, and validation of essential analytical assays, including the Bicinchoninic Acid (BCA) assay, dot blot immunoassays, and ELISA, to support formulation screening and characterization.

# Objectives

1. Develop and optimize formulations for encapsulating therapeutic proteins, such as Immunoglobulin G (IgG), within polymer microspheres.

2. Evaluate the effects of various excipients, polymers, and formulation parameters on key characteristics including solubility, stability, loading efficiency, and release profile.

3. Establish and validate analytical methods, including BCA, ELISA, and dot blot assays, for quantifying protein concentration and assessing retained biological activity.

4. Characterize the physical properties of formulations, such as particle morphology, porosity, and viscosity, to ensure suitability for the intended application.

# Methodology

Blank poly(lactic-co-glycolic) acid (PLGA) microspheres were formulated to investigate the effects of polymer concentration on particle morphology. Expansorb PO26 polymer was used at concentrations ranging from 1% to 20% (w/v) in dichloromethane (DCM). The resulting particles were characterized using ZOE microscopy for size and density and Scanning Electron Microscopy (SEM) for surface topography and internal structure. Formulations with lower PLGA concentrations, such as MA15 (10%), exhibited more jagged surface features, hypothesized to enhance subsequent protein adsorption.

# Results

## Effect of PLGA Concentration on Microsphere Morphology

Blank microspheres were formulated with varying concentrations of poly(lactic-co-glycolic) acid (PLGA) to assess the impact on particle morphology. Five formulations (MA14–MA18) were prepared with PLGA concentrations of 20%, 10%, 5%, 2%, and 1% (w/v) in dichloromethane (DCM).

Microscopic analysis revealed that PLGA concentration directly influenced microsphere characteristics. The highest PLGA concentration (MA14, 20%) produced microspheres of uniform size and shape with high yield. Lower PLGA concentrations resulted in greater size dispersity, less uniform shapes, and visually denser particles, with the lowest yields observed at 1% and 2% PLGA [FIGURE 1].

**Figure 1: Light microscopy images of microspheres formulated with decreasing PLGA concentrations. From left to right: MA14 (20%), MA15 (10%), MA16 (5%), MA17 (2%), and MA18 (1%).**

Scanning electron microscopy (SEM) was used to examine surface and internal morphology. The MA14 formulation (20% PLGA) exhibited a relatively smooth but porous surface [FIGURE 2]. The MA15 formulation (10% PLGA) showed a jagged surface appearance [FIGURE 3]. Cross-sectional analysis of the MA16 formulation (5% PLGA) revealed a porous internal structure [FIGURE 4].

**Figure 2: SEM image of an MA14 microsphere (20% PLGA) showing a porous surface.**

**Figure 3: SEM image of an MA15 microsphere (10% PLGA) showing a jagged surface texture.**

**Figure 4: SEM cross-section of an MA16 microsphere (5% PLGA) showing internal porosity.**

## Most Impactful Features

\* Higher PLGA concentration (20%) produced more uniform and smoother microspheres. Lower concentrations led to increased surface irregularity and size dispersity.

## Effect of Polymer Type and Dispersed Phase Ratio on Microsphere Morphology

The effect of polymer type and the ratio of aqueous to oil phase within the dispersed phase on microsphere morphology was investigated. Two different Innocore polymers (1 and 4) were used to prepare formulations MA258-265. For each polymer, the aqueous-to-oil phase ratio was varied at 1:2, 1:4, 1:8, and 1:16. Post-lyophilization, microscopic images showed that increasing the proportion of the oil phase resulted in visibly smaller and more uniform microspheres for both Innocore 1 and Innocore 4 polymers [FIGURE 5, FIGURE 6, FIGURE 7, FIGURE 8].

**Figure 5: MA258 microspheres (Innocore 1) with a 1:2 aqueous-to-oil ratio.**

**Figure 6: MA261 microspheres (Innocore 1) with a 1:16 aqueous-to-oil ratio, showing smaller and more uniform particles compared to the 1:2 ratio.**

**Figure 7: MA262 microspheres (Innocore 4) with a 1:2 aqueous-to-oil ratio.**

**Figure 8: MA265 microspheres (Innocore 4) with a 1:16 aqueous-to-oil ratio, showing a similar trend of size reduction and improved uniformity.**

## Most Impactful Features

\* Increasing the oil phase volume relative to the aqueous phase (e.g., from 1:2 to 1:16) consistently produced smaller and more uniform microspheres, independent of the Innocore polymer type used.

## Excipient Solubility and Dispensing Calibration

The SDB-1 solid dispenser was calibrated for various excipients. Using a disk size of 2 and vibration settings of 2 or 3, most materials were dispensed with an accuracy within 1 mg of the target value. Sodium Oleate and Dextran Sulfate 500k MW could not be dispensed reliably [TABLE 1].

|  |  |  |  |
| --- | --- | --- | --- |
| Material | SDB1 Disk Size | Vibration | Amount Dispensed (mg) |
| Povidone K-30 | 2 | 2 | 2.6 |
| Benzethonium Chloride | 2 | 2 | 5.2 |
| Sodium Oleate | 2 | 2 | Didn't work repeatedly |
| Trehalose | 2 | 2 | 6.1 |
| Sodium Alginate | 2 | 3 | 7 |
| Sorbitol | 2 | 2 | 4 |
| Ethyl Cellulose 48% Ethoxy Content | 2 | 2 | 3.6 |
| Arginine | 2 | 2 | 5.7 |
| Dextran Sulfate 500k MW | 2 | 2 | Didn't work repeatedly |
| Glycine | 2 | 2 | 6 |
| Aqualon CMC | 2 | 2 | 5 |
| Ammonium Sulfate | 2 | 2 | 7 |
| PEG 3350 | 2 | 2 | 4.5 |
| Polysorbate 80 |  |  | 100 µL |
| Castor Oil |  |  | 100 µL |

**Table 1: SDB-1 solid dispenser calibration settings and dispensed amounts for various excipients.**

A qualitative solubility screen of these excipients was performed in a panel of eight solvents. Polysorbate 80, a liquid, was fully soluble in all tested solvents. Povidone K-30 and Ethyl Cellulose showed broad solubility in most organic solvents and aqueous solutions but were only partially soluble in acetonitrile. Aqualon CMC was soluble only in PBS and water. The complete solubility profile is detailed in [TABLE 2].

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Material | Amount | Acetonitrile | Ethanol | NMP | DMSO | DCM | Acetone | PBS | Water |
| Povidone K-30 | ~2.6 mg | Partial | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Benzethonium Chloride | ~5.2 mg | Partial | No | Yes | Yes | Yes | Yes | Partial | No |
| Sodium Oleate | 5.9-11.5 mg | - | - | - | - | No | Yes | Partial | Yes |
| Trehalose | ~6.1 mg | No | Partial | Yes | Yes | Yes | No | Yes | Yes |
| Sodium Alginate | ~7 mg | No | No | Partial | Partial | Partial | No | Yes | Yes |
| Sorbitol | ~4 mg | No | No | Yes | Yes | No | No | Yes | Yes |
| Ethyl Cellulose | ~3.6 mg | Partial | No | Yes | Yes | Yes | Yes | No | No |
| Arginine | ~5.7 mg | No | No | Partial | Partial | No | Yes | Yes | Yes |
| Glycine | 6 mg | No | No | Partial | No | No | Partial | Yes | Yes |
| Aqualon CMC | 5 mg | No | No | No | No | No | No | Yes | Yes |
| Ammonium Sulfate | ~7 mg | No | No | Yes | Partial | Partial | Partial | Yes | Yes |
| PEG 3350 | ~4.5 mg | Yes | No | Partial | Partial | Yes | Partial | Yes | Yes |
| Polysorbate 80 | 100 µL | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Castor Oil | 100 µL | No | Yes | Yes | Yes | Yes | Yes | No | No |

**Table 2: Solubility of excipients in various solvents. "Yes" indicates full solubility, "No" indicates insolubility, and "Partial" indicates partial solubility.**

## Screening of Additives for Compatibility and Viscosity

Thirteen potential additives were screened for compatibility with dichloromethane (DCM) and polyvinyl alcohol (PVA). Upon addition of PVA, the solution containing Ethyl Cellulose (10 mg/mL) immediately became cloudy, indicating precipitation or incompatibility. An absorbance scan of this turbid solution from 230 to 600 nm identified a maximum absorbance peak at 230 nm [FIGURE 9].

**Figure 9: Absorbance scan of the Ethyl Cellulose solution after PVA addition, showing a peak at 230 nm.**

A subsequent absorbance scan of all additive-containing wells at 230 nm showed that Poly-L-Lysine, Polyethyleneimine, Polysorbate 80, and Ethyl Cellulose produced the highest absorbance readings [TABLE 3].

|  |  |  |
| --- | --- | --- |
| Well | Additive (10 mg/mL\*) | Absorbance at 230 nm |
| A1 | Ammonium Sulfate | 0.120 |
| A2 | Poly-L-Lysine | 0.963 |
| A3 | Polyethyleneimine | 0.521 |
| A4 | Polysorbate 80 | 0.457 |
| A5 | Alginic Acid | 0.088 |
| A6 | Dextran Sulfate | 0.124 |
| A7 | Heparin | 0.113 |
| A8 | Polyacrylamide | 0.101 |
| A9 | Chitosan | 0.106 |
| A10 | Polyallylamine | 0.118 |
| A11 | Arginine | 0.122 |
| A12 | Ethyl Cellulose | 0.525 |
| B1 | Sodium Hyaluronate\* | 0.102 |
| B2 | Blank (DI H2O) | 0.091 |

**Table 3: Absorbance readings at 230 nm for additives after addition of DCM and PVA. \*Sodium Hyaluronate was tested at 5 mg/mL.**

A qualitative viscosity assessment was performed on additives that were difficult to aspirate. Polyacrylamide, Chitosan, and Sodium Hyaluronate were highly viscous at their initial stock concentrations. Serial dilutions were performed to identify the concentration at which they became free-flowing enough for pipetting [TABLE 4].

|  |  |  |  |
| --- | --- | --- | --- |
| Additive | Initial Concentration | Observation | Free-Flowing Concentration |
| Polyacrylamide | 10 mg/mL | Remained viscous | > 5 µg/mL |
| Chitosan | 10 mg/mL | Viscous | 0.078 mg/mL |
| Sodium Hyaluronate | 5 mg/mL | Viscous | 0.156 mg/mL |

**Table 4: Viscosity assessment of selected additives and the concentrations at which they became free-flowing.**

## Validation of Protein Quantification Assays

The Bicinchoninic Acid (BCA) assay was validated for use with PLGA microspheres and different protein payloads. The potential for interference from blank PLGA microspheres (MA14) was tested. A serial dilution of blank spheres (1.25 mg/mL to 0.15625 mg/mL) in DMSO was assayed. The resulting absorbance values were low (0.2028 at the highest concentration) and decreased with dilution. The absorbance at the lowest sphere concentration (0.0945) was nearly identical to the blank control (0.0836), confirming minimal interference from the polymer matrix.

The BCA assay was also validated for quantifying enzymes. Standard curves were generated for Horseradish Peroxidase (HRP) and Alkaline Phosphatase (APT) via 2x serial dilutions starting from 0.625 mg/mL. Both enzymes produced linear standard curves with high coefficients of determination (R² > 0.99), demonstrating the suitability of the BCA assay for their quantification [FIGURE 10, FIGURE 11].

**Figure 10: HRP standard curve generated using the BCA assay, showing a linear response (R² = 0.998).**

**Figure 11: APT standard curve generated using the BCA assay, showing a linear response (R² = 0.994).**

## Post-Loading of IgG into Blank Microspheres

Initial attempts to post-load human IgG (hIgG) into pre-formed blank microspheres (MA14 and MA16) were unsuccessful. Varying incubation times (1, 2, and 7 days) and hIgG concentrations (0.2, 1, and 5 mg/mL) did not result in any significant loading.

A subsequent experiment screened 21 different excipients for their ability to enhance hIgG post-loading into MA15 blank microspheres. Each excipient was added at a final concentration of 1 mg/mL to a suspension of 10 mg of spheres and 1 mg/mL of hIgG. Loading was quantified using the BCA assay after a 24-hour incubation. Several excipients produced background noise in the BCA assay, confounding the results for those formulations. Of the non-interfering excipients, Chitosan and Polyallylamine HCl resulted in the highest hIgG loading, at 3.74% and 2.57% respectively. A summary of the results is presented in [TABLE 5].

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Formulation # | Excipient | BCA Background Noise? | hIgG Loading | Improved Loading? |
| 1 | Alginic acid | N | 1.48% | Y |
| 2 | Ammonium sulfate | N | 1.04% | N |
| 3 | Castor oil | Y | 1.10% | ? |
| 4 | Dextran 40 | N | 1.14% | N |
| 5 | Distearoyl-Phosphocholine | N | 1.44% | Y |
| 6 | Polyallylamine, HCl | N | 2.57% | Y |
| 7 | Polysorbate 80 | Y | 1.84% | ? |
| 8 | Arginine, HBr | N | 0.99% | N |
| 9 | Benzethonium chloride | Y | 3.82% | ? |
| 10 | Chitosan | N | 3.74% | Y |
| 11 | Lecithin 4C | N | 2.64% | Y |
| 12 | Polyacrylamide | N | 1.56% | Y |
| 13 | Polyethyleneimine | Y | 1.06% | N |
| 14 | Povidone (PVP) K30 | N | 1.21% | Y |
| 15 | Agarose, low gelling point | N | 1.89% | Y |
| 16 | Dextran sulfate | N | 1.07% | N |
| 17 | Ethyl cellulose | N | 1.96% | Y |
| 18 | Heparin 4C | N | 1.09% | N |
| 19 | Poly-L-Lysine 20C | Y | 1.80% | ? |
| 20 | Sodium hyaluronate 4C | N | 1.18% | N |
| 21 | Span 80 | Y | 1.08% | N |

**Table 5: Effect of various excipients on post-loading of hIgG into blank microspheres.**

## Most Impactful Features

\* Chitosan — Increased hIgG loading to 3.74% without interfering with the BCA assay.

\* Polyallylamine HCl — Increased hIgG loading to 2.57% without assay interference.

\* Lecithin — Increased hIgG loading to 2.64% without assay interference.

## Detection of Encapsulated Enzyme and Antibody Activity

The activity of encapsulated payloads was assessed using colorimetric and immunological assays. The presence of active alkaline phosphatase released from MA10 and MA12 microspheres was tested using a BCIP/NBT substrate assay. After a 30-minute incubation, a slight indigo color change was observed in both the MA10 and MA12 solutions, indicating the presence of active enzyme. No color change was seen in the Blank 12 microsphere negative control. Indigo-stained microspheres were visible as a pellet in the MA10 and MA12 tubes [FIGURE 12].

**Figure 12: BCIP/NBT assay results. From left to right: MA10, MA12, and Blank 12 (negative control). A visible indigo color indicates positive detection of alkaline phosphatase in MA10 and MA12.**

The retained activity of encapsulated Goat Anti-Mouse IgG released from MA35 microspheres was measured using a dot blot immunoassay. The optimal concentration of the secondary antibody (Biotinylated Rabbit Anti-Goat) was determined to be a 1:500 dilution. The supernatant from the MA35 sample, with a protein concentration of 0.614 µg/mL, produced a signal intensity that was visually comparable to the 0.625 µg/mL and 0.3125 µg/mL standards, indicating that the encapsulated antibody retained its activity [FIGURE 13].

**Figure 13: Dot blot membrane for Goat Anti-Mouse IgG activity. The sample (membrane #6) shows signal intensity between the 0.625 µg/mL (membrane #3) and 0.3125 µg/mL (membrane #4) standards.**

## ELISA Optimization and Analysis of Microsphere Samples

An ELISA was developed to quantify antibody release. Initial optimization experiments evaluated various concentrations of detection reagents, settling on 1:10,000 b-RxG and 1:5,000 SAV HRP for subsequent sample analysis. An earlier iteration of the assay using a different antibody combination showed non-specific binding to the human IgG negative control down to a concentration of 0.01 mg/mL.

The optimized ELISA was used to measure IgG released from MA228 microspheres that had been incubated at different temperatures. Samples were collected after two days (37°C) and after a weekend (RT, 37°C, 4°C). Absorbance readings for the samples and standards are presented in [TABLE 6]. A subsequent run with adjusted standard concentrations was performed to improve quantification in the lower range [FIGURE 14].

|  |  |  |
| --- | --- | --- |
| Column | Sample/Standard | Absorbance |
| 8 | MA228 37°C (4/12) | 0.407 |
| 9 | MA228 RT (4/15) | 0.174 |
| 10 | MA228 37°C (4/15) | 0.168 |
| 11 | MA228 4°C (4/15) | 0.167 |
| GxM Std | 10 µg/mL | 0.395 |
| GxM Std | 8 µg/mL | 0.329 |
| GxM Std | 6 µg/mL | 0.279 |
| GxM Std | 4 µg/mL | 0.222 |
| GxM Std | 2 µg/mL | 0.163 |
| GxM Std | 1 µg/mL | 0.133 |
| GxM Std | 0 µg/mL | 0.098 |

**Table 6: ELISA absorbance readings at 450 nm for MA228 release samples and Goat-anti-Mouse (GxM) standards.**

**Figure 14: Representative ELISA plate result showing color development for mIgG standards (columns 1-7), GxM standards with a refined concentration range of 0-1 µg/mL (rows A-G), and MA228 samples (columns 8-9).**

# Conclusion

This investigation established foundational methods for microsphere formulation, protein loading, and analytical characterization. Microsphere morphology, including surface porosity and particle density, was tunable by modulating the poly(lactic-co-glycolic) acid (PLGA) concentration. Lower PLGA concentrations produced particles with more jagged surface features, which may enhance subsequent protein loading. Further formulation work demonstrated that varying the aqueous-to-oil phase ratio with different Innocore polymers influences final particle characteristics.

Protein loading studies revealed that post-incubation of blank microspheres in immunoglobulin G (IgG) solutions was ineffective. However, the inclusion of excipients significantly improved loading efficiency. A screen of 21 additives identified Chitosan, Polyallylamine HCl, and Lecithin as the most effective candidates for enhancing IgG uptake into blank MA15 microspheres without interfering with the quantification assay. Some additives, such as Ethyl Cellulose, were found to be incompatible with polyvinyl alcohol (PVA), while others like Polyacrylamide were determined to be too viscous for practical application at the tested concentrations.

A suite of analytical methods was validated for these formulations. The bicinchoninic acid (BCA) assay was confirmed to be a reliable method for protein quantification, demonstrating minimal interference from the blank PLGA matrix and producing linear standard curves for model enzymes like horseradish peroxidase (HRP) and alkaline phosphatase (R² > 0.99). Enzyme and antibody activity was preserved post-encapsulation, as confirmed by a BCIP/NBT colorimetric assay for alkaline phosphatase and a dot blot immunoassay for Goat Anti-Mouse IgG. The dot blot method requires further optimization to eliminate non-specific binding observed with the human IgG negative control.